CYTOTOXICITY ASSAY

Field of the Invention

The present invention relates to methods for detecting CTL activity using flow cytometry to detect cleavage of cytotoxicity-related enzymes.

Background of the Invention

The ^{51}Cr-release assay has been the gold standard to measure CTL activity during an immune response for over 3 decades. However, the assay has a number of technical limitations and recent data has brought into question the physiological relevance of the ^{51}Cr-release assay and whether it is a true measure of CTL activity measured against target cells *in vivo*, especially in solid tumors. The lysis of target cells in *in vitro* CTL assays can be induced via two distinct mechanisms: 1) A membranolysis process mediated when perforin is simply inserted into the target cell membrane; and, 2) DNA fragmentation (apoptosis) induced by the action of Granzyme B and Granzyme A entering a target cell via perforin and/or vesicle fusion events at the target cell surface. The ^{51}Cr-release assay measures the first of these two read-outs. The latter mechanism has more physiologic relevance and is required *in vivo* for target cell killing. Recent observations indicate that measurement of effector cell cytotoxicity by the ^{51}Cr-release assay may not be accurate or in some cases, even relevant. Based on these observations, the investigator must remain cognizant of the fact that effector cells determined to be cytotoxic based on ^{51}Cr-release assays may indeed not be CTL capable of tumor cell killing via DNA fragmentation *in vivo*. This may account for many of the results in the cancer immunotherapy literature in which effector cell responses monitored *in vitro* may not correlate with vaccine efficacy in the patient. For example, peptide vaccines selected by virtue of positive effector cell cytotoxicity date generated using ^{51}Cr-release assays may not accurately reflect the actual epitopes active in inducing CTL lysis *in* vivo via DNA fragmentation. In addition, there are a number of other issues plaguing the use of ^{51}Cr-based assays, including the need to use hazardous radioactivity, and problems related to the use of scintillation counters.

The JAM assay is a newer method that solves some of the issues with ^{51}Cr-release. Although it measures DNA fragmentation effectively, it has serious limitations due to the need to label the DNA of target cells with agents such as ^{3}H-thymidine or ^{125}I-deoxyuridine. This method invariably causes DNA damage and

changes the physiology of the target cell before the CTL assay. In addition, the ^{3}H-thymidine labelling method does not afford the same degree of sensitivity as ^{51}Cr and ^{125}I is a dangerous agent requiring considerable lead shielding of both the lab worker as well as the lab area. Overall, a non-radioactive method to measure apoptosis induction and DNA fragmentation in target cells during CTL attack would be a more ideal assay, especially when contemplating its use in clinical trial monitoring.

Detection of capsase cleavage using fluorogenic caspase substrates has also been used to demonstrate cytotoxic T cell activity (Liu, et al. Visualization and quantification of T cell-mediated cytotoxicity using cell-permeable fluorogenic caspase substrates. Nature Medicine, Jan. 2003, 9(1):4-5.) In addition, other highly sensitive methods to enumerate antigen-specific $CD8^{+}$ T-cells have been introduced, such as intracellular cytokine staining, the ELISPOT assay, and staining for T-cells using class I MHC tetramers. However, these assays do not measure ultimate cytolytic function, which is especially important in analyzing anti-tumor immune responses. Assays offering a suitable levels of sensitivity, specificity, safety and ease-of-use are lacking. A new and sensitive assay system for detecting CTL activity is provided herein.

Summary of the Invention

The present invention provides a cytotoxicity assay that detects DNA fragmentation- and/or apoptosis-related changes in target cells following contact with effector cells. In one embodiment, the assay is conducted using one or more reagents that detect cleavage of a caspase or phosphorylation of histones. In a preferred embodiment, cleavage of caspase is detected using a monoclonal antibody. In another preferred embodiment, phosphorylation of histones is detected using a monoclonal antibody. In other embodiments, cleaved caspase and/or phosphorylated histones are detected using at least one monoclonal antibody coupled with flow cytometric analysis.

Brief Description of the Drawings

Fig. 1. Stability of target cell labeling dye and gating strategy developed for monitoring CTL activity using the caspase 3-cleavage in target cells. A. DDAO-SE cell tracker dye brightly and stably labels CTL target cell lines. Shown is staining of a number of commonly used suspension-cultured and adherent cell lines: P815,

EL4, T2 lymphoma, NIH 3T3, 4T1 breast cancer, and B16F10 melanoma. Cells were stained with DDAO-SE and incubated for the indicated time intervals (0, 1, 3, or 15 h) and analyzed by FACS. The mean fluorescent intensity and the coefficient of variation (CV) are shown for each cell line. The result of one out of two similar experiments is shown. B. Gating strategy used to enumerate caspase 3-cleavage in DDAO-SE-labeled targets. An example of DDAO-SE-labeled P815 cells with C57BL/6 anti-Balb/c MLR cells is shown. The first plot shows FSC versus SSC with gating of the entire population (R1). Gate R1 cells were then analyzed using SSC and FSC versus FL4 fluorescence (DDAO-SE positive). DDAO-SE positive cells were gated as shown (R2 and R3) excluding low FSC (dead) cells. R2*R3 cells were then analyzed using FL2 (PE-labeled cleaved caspase 3) versus FL4 (DDAO-SE) to determine the percentage of apoptotic targets (R4).

Fig. 2. Measurement of CTL activity in murine MLR using the caspase 3 cleavage assay and dependence of caspase 3 cleavage in the target on perforin and granzyme secretion by CTL. C57BL/6 anti-Balb/c or Balb/c anti-C57BL/6 MLR effector cells were incubated with labeled P815 targets (A) for different time intervals (0.5, 1, 2, and 3 h) followed by staining for caspase 3 cleavage as shown. Caspase 3 cleavage was found only with the anti-Balb/c MLR effectors. B. P815 or EL4 targets labeled with DDAO-SE were incubated with C57BL/6 anti-Balb/c or Balb/c anti-C57BL/6 MLR effectors for different time intervals as shown. The percentage caspase 3-cleaved target cells is shown. The results show the specificity of the assay with MLR effectors only killing the H-2 unmatched targets. The results are representative of 3 experiments with similar results. C. Concanamycin A, an inhibitor of cytolytic granule secretion by CTL prevents caspase 3 cleavage in P815 targets incubated with C57BL/6 anti-Balb/c MLR effector cells. Five-day MLR cultures were isolated and diluted with naïve syngeneic C57BL/6 spleen lymphocytes at a 1:1, 1:3, 1:9 or with no dilution, as shown. The diluted effectors were incubated with labeled P815 cells. Controls were naïve C57BL/6 spleen cells with no MLR cells or labeled P815 cells alone. The assays were stained for caspase 3-cleavage after 3 h.

Fig. 3. Comparison of the caspase 3 cleavage assay and ^{51}Cr-release for monitoring CTL activity using murine MLR effectors and CTL isolated after

peptide-based vaccine responses in mice. A. Effector cells from a murine MLR (b→d) were incubated with 100,000 P815 or EL4 target cells (control) in a 4 h assay at the indicated E:T ratios. For the ^{51}Cr-release assay, we started at a 16:1 dilution and diluted down to 0.2:1, while the caspase 3 cleavage assay was tested between 1:1 and 0.1:1 E:T ratio. The results of 1 out of 2 similar experiments are shown. B. Caspase 3 cleavage assay is equally reliable and specific as the ^{51}Cr-release assay for monitoring recall responses from peptide-immunized mice. Mice were immunized with the melanoma TRP2 peptide and tested at the indicated E:T ratios.

Fig. 4. Monitoring CD8$^+$ T-cell responses following viral vector-based vaccination in mice using the caspase 3-cleavage CTL assay yields comparable results as IFN-γ ELISPOT analysis. HLA-A2.1/K^b transgenic mice were immunized with ALVAC-gp100 and spleen cells re-stimulated for 5 days with a pool of gp100 peptides. Viable cells were isolated and tested for CTL activity using caspase 3 cleavage with at a 20:1 E:T ratio (left panel), or for IFN-γ production in ELISPOT assays (right panel) at 100,000 cells per well. Targets were P815-A2/K^b transfectants pulsed with gp100 peptides or a control A2-binding CMV pp65 peptide. The results of two independent experiments are shown. Points represent results from individual vaccinated mice.

Fig. 5. Monitoring of antigen-specific human CD8$^+$ T cell responses using the caspase 3-cleavage assay. Primary peptide-specific human T-cell lines were generated from PBMC of HLA-A*0201$^+$ donors using peptide-pulsed autologous DC and activated B cells as described. T-cell underwent four rounds of peptide-specific stimulation before being tested for CTL activity. A. Application of the caspase 3-cleavage assay to foreign antigen-specific (HIV rev peptide) T cell lines. T cells were harvested and incubated with DDAO-SE-labeled T2 lymphoma targets pulsed with the HIV rev or CMV pp65 peptides, or with no peptide. The T cells were diluted with naïve autologous PBMC at a 1:1 or 1:5 ratio before mixing with 100,000 pulsed target at a 1:1 ratio. The cells were incubated for 1 or 3 h, as indicated, and stained for cleaved caspase 3. Target cells alone ("T alone") were also used as controls. Results of one out of 3 similar experiments are shown. B. CTL activity measured in self-antigen-specific (CEA CAP1 peptide) human T-cell lines using caspase 3 cleavage or

Annexin-V plus 7-AAD staining assays. Effector cells were diluted at the indicated ratios with naïve autologous PBMC before mixing with peptide-pulsed T2 cells pulsed with the CAP1 specific peptide or non-specific CMV pp65 peptide. Target cells alone ("T alone") and naïve PBMC from the donor were used as negative controls. The results of 3 h incubation are shown. Similar results were obtained after 1 or 4 h incubation (not shown). Results of one of out two similar experiments are shown.

Fig. 6. The caspase 3-cleavage is a highly sensitive assay for monitoring murine CTL activity. Spleen cells from b→d MLR were harvested and incubated with DDAO-SE-labeled P815 target cells. The MLR effector cells were diluted with naïve syngeneic spleen cells at the ratios indicated before mixing with the P815 targets at a 1:1 ratio. One hundred thousand diluted effector cells and 100,000 target cells were in the assay. The upper panel (A) shows dot plots for DDAO-SE versus caspase 3-cleavage after FACS analysis at different dilutions of the MLR effector cells. Naïve B6 spleen cells mixed with target cells are shown as a negative control. B. Results of two independent experiments showing caspase 3 cleavage at the indicated b→d MLR effector cell dilutions. The effectors and P815 target cells were incubated for 2 or 4 h before staining for cleaved caspase 3.

Fig. 7. The caspase 3-cleavage is a highly sensitive assay for monitoring human $CD8^+$ T-cell activity with a similar level of sensitivity as TCR staining with HLA-peptide pentamers. HLA-A*0201-restricted HIV gag peptide-specific human T-cell lines were generated as before and tested for CTL activity. The T cells were diluted with naïve autologous PBMC at the ratio indicated before adding at a 1:1 ratio with HIV or CMV (specificity control) peptide-pulsed T2 targets. Targets alone and naïve PBMC with no effector cells were used as negative controls. A. Dot plots show representative results when highly diluted HIV gag peptide-specific T cells are incubated T2 cells pulsed with HIV gag peptide or non-specific CMV pp65 peptide. Significant differences in percent caspase cleavage were routinely detected even at the 1:199 dilution. B. Bar graph showing the average and standard deviation of three separate experiments testing the caspase 3 cleavage assay to measure CTL activity in HIV gag peptide-specific T cells lines diluted with naïve PBMC as indicated. In each case, 150,000 diluted effectors were incubated with 150,000 DDAO-SE-labeled,

peptide-pulsed targets. As a comparison, the same T cells were stained with an HLA-gag peptide pentamer (ProImmune, Oxford, UK) in parallel (C). A CMV pp65 peptide-specific T-cell line was used as the specificity control. In order to compare the pentamer assay to the caspase 3-cleavage assay, 150,000 gated T cells were collected for each data point (the same amount of effector cells added to the CTL assay).

Detailed Description

The present invention relates to reagents and assays for performing cytotoxicity assays. The assays are suitable to detecting cytotoxicity mediated by immune effector cells including but not limited to T cells, natural killer (NK) cells, granulocytes, monocytes, macrophages, and the like. The assays are generally based on the detection of DNA fragmentation- and/or apoptosis-related changes in a target cell. For instance, in certain embodiments, cleavage of an enzyme involved in the apoptotic process is detected. In another, a change in the characteristic of a DNA-associated protein during the apoptotic process is detected. Such changes are suitable to detection using devices that measure cell fluorescence, such as a Fluorescence Activated Cell Sorter (FACS) machine or flow cytometer.

In one embodiment, the present invention relates to an assay for measuring the cleavage of one or more caspases during the apoptotic process. Caspases shown to play a role in apoptosis that may be suitable for detection in an assay as shown herein many include but are not limited to caspase 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and 14. In one embodiment, caspase 3 is detected using the assay described herein. Another enzyme known to be cleaved during the apoptotic process is poly-ADP ribose polymerase (PARP). Cleavage of caspases, in particular caspase 3, and PARP are early events during apoptosis and are triggered in CTL targets. In addition to the cleavage of caspases and PARP, a number of intracellular substrates are phosphorylated during the induction of apoptosis. For instance, histone H2A.X is phosphorylated in wide variety of cell lineages. Antibodies with specific binding capacity for cleaved caspases, as well as antibodies specific for phosphorylated histone H2.AX are commercially available and suitable for use in practicing the present invention.

In certain embodiments, dyes are utilized to stain the target cells prior to contact with effector cells. Suitable dues include Vial T, CFSE, and DDAO-SE, each

of which are known in the art. A preferred dye is DDAO-SE (Molecular Probes). Other suitable dyes are also known in the art and may be useful in practicing the present invention.

In one embodiment, the assay of the present invention provides is carried out by: 1) preparing effector cells (E); 2) preparing target cells (T), including tagging the cells with a detectable marker such as a fluorescent dye; 3) mixing the effector cells and the target cells at a sufficient number of E:T ratios; 4) incubating the mixture for a sufficient period of time and under conditions suitable for at least some of the effector cells to become cytotoxic toward at least some of the target cells; 5) fixing and permeabilizing the mixed cells; 6) staining the cells with a detectable reagent having the ability to bind a protein or fragment thereof that undergoes a change during or results from the apoptotic process; and, 7) detecting the detectable reagent. In certain embodiments, the detectable reagent is detectable by virtue of a fluorescent tag or moiety contained within the reagent or by detecting a secondary reagent that binds to the detectable reagent, the secondary reagent being detectable by virtue of a fluorescent tag or moiety contained within the secondary reagent. In preferred embodiments, the detectable reagent and / or the secondary reagent is an antibody, which may be fluorescently labeled. The detectable reagent may detected by any of several well-known methods including but not limited to FACS. In one preferred embodiment, the detectable reagent is an anti-Caspase 3 antibody (such as the PE Conjugated Monoclonal Rabbit Anti-Active Caspase 3 Antibody, BD Pharmingen Catalog No. 550914). Other suitable reagents are known in the art.

In certain embodiments, it may be beneficial or desirable to contact or "pulse" a target cell with a peptide to which the effector cells may respond prior to staining the target cells and / or mixing the target and effector cells. For instance, a target cell may be pulsed with a peptide corresponding to the amino acid sequence of an infectious agent such as HIV or a tumor antigen. Suitable tumor antigens include include, for example, gp100 (Cox et al., *Science*, 264:716-719 (1994)), MART-1/Melan A (Kawakami et al., *J. Exp. Med.*, 180:347-352 (1994)), gp75 (TRP-1) (Wang et al., *J. Exp. Med.*, 186:1131-1140 (1996)), tyrosinase (Wolfel et al., *Eur. J. Immunol.*, 24:759-764 (1994)), NY-ESO-1 (WO 98/14464; WO 99/18206), melanoma proteoglycan (Hellstrom et al., *J. Immunol.*, 130:1467-1472 (1983)), MAGE family antigens (i.e., MAGE-1, 2,3,4,6, and 12; Van der Bruggen et al., *Science*, 254:1643-1647 (1991); U.S. Pat. Nos. 6,235,525), BAGE family antigens

(Boel et al., *Immunity*, 2:167-175 (1995)), GAGE family antigens (i.e., GAGE-1,2; Van den Eynde et al., *J. Exp. Med.*, 182:689-698 (1995); U.S. Pat. No. 6,013,765), RAGE family antigens (i.e., RAGE-1; Gaugler et at., *Immunogenetics*, 44:323-330 (1996); U.S. Pat. No. 5,939,526), N-acetylglucosaminyltransferase-V (Guilloux et at., *J. Exp. Med.*, 183:1173-1183 (1996)), p15 (Robbins et al., *J. Immunol.* 154:5944-5950 (1995)), ß-catenin (Robbins et al., *J. Exp. Med.*, 183:1185-1192 (1996)), MUM-1 (Coulie et al., *Proc. Natl. Acad. Sci.* USA, 92:7976-7980 (1995)), cyclin dependent kinase-4 (CDK4) (Wolfel et al., *Science*, 269:1281-1284 (1995)), p21-*ras* (Fossum et at., *Int. J. Cancer*, 56:40-45 (1994)), BCR-*abl* (Bocchia et al., *Blood*, 85:2680-2684 (1995)), p53 (Theobald et al., *Proc. Natl. Acad. Sci.* USA, 92:11993-11997 (1995)), p185 HER2/neu (erb-B1; Fisk et al., *J. Exp. Med.*, 181:2109-2117 (1995)), epidermal growth factor receptor (EGFR) (Harris et al., Breast Cancer Res. Treat, 29:1-2 (1994)), carcinoembryonic antigens (CEA) (Kwong et al., *J. Natl. Cancer Inst.*, 85:982-990 (1995) U.S. Pat. Nos. 5,756,103; 5,274,087; 5,571,710; 6,071,716; 5,698,530; 6,045,802; EP 263933; EP 346710; and, EP 784483); carcinoma-associated mutated mucins (i.e., MUC-1 gene products; Jerome et al., *J. Immunol.*, 151:1654-1662 (1993)); EBNA gene products of EBV (i.e., EBNA-1; Rickinson et al., *Cancer Surveys*, 13:53-80 (1992)); E7, E6 proteins of human papillomavirus (Ressing et al., *J. Immunol*, 154:5934-5943 (1995)); prostate specific antigen (PSA; Xue et al., *The Prostate*, 30:73-78 (1997)); prostate specific membrane antigen (PSMA; Israeli, et al., *Cancer Res.*, 54:1807-1811 (1994)); idiotypic epitopes or antigens, for example, immunoglobulin idiotypes or T cell receptor idiotypes (Chen et al., *J. Immunol.*, 153:4775-4787 (1994)); KSA (U.S. Patent No. 5,348,887), kinesin 2 (Dietz, et al. Biochem Biophys Res Commun 2000 Sep 7;275(3):731-8), HIP-55, TGFβ-1 anti-apoptotic factor (Toomey, et al. Br J Biomed Sci 2001;58(3):177-83), tumor protein D52 (Bryne J.A., et al., Genomics, 35:523-532 (1996)), H1FT, NY-BR-1 (WO 01/47959), NY-BR-62, NY-BR-75, NY-BR-85, NY-BR-87 and NY-BR-96 (Scanlan, M. Serologic and Bioinformatic Approaches to the Identification of Human Tumor Antigens, in *Cancer Vaccines 2000*, Cancer Research Institute, New York, NY), among others, including wild-type, modified, mutated versions thereof.

In certain embodiments, the time during which the target and effector cells are incubated may be modified. For instance, it may be desirable to incubate the mixture for one, two, three, four, five, six or more hours prior to further analysis.

Other aspects of the present invention include kits for carrying out the assays described herein. A kit may include materials useful in preparing effector cells, a suitable number of effector cells, a fluorescent dye with which to stain the target cells, a device such as a 96-well plate in which the target and effector cells may be mixed and incubated, materials required for fixing and permeabilizing the cells, a detectable reagent, and / or a secondary reagent. Different combinations of such materials may be organized as a kit in order to aid the skilled artisan in carrying out the assay of the present invention.

A better understanding of the present invention and of its many advantages will be had from the following examples, given by way of illustration.

EXAMPLES

Example 1

MATERIALS AND METHODS

A. Reagents and cell culture media

Anti-cleaved caspase 3 (reactive against both human and mouse proteins) was phycoerythrin (PE)-labeled and purchased from BD Biosciences (Mississauga, ON). Anti-phospho-histone H2A.X-FITC was purchased from Upstate Cell Signaling Solutions (Lake Placid, NY). The cell tracker dye, DDAO-SE was obtained from Molecular Probes (Eugene, OR). All cell culture medium was from Invitrogen (Mississauga, ON). Cytokines (IL-2, IL-2, IL-12, and IL-6) were from R&D Systems (Minneapolis, MN). Anti-IFN-γ antibody pairs for human and mouse IFN-γ ELISPOT analysis was from MabTech (Amsterdam, NL) and BD Biosciences (Mississauga, ON), respectively. ^{51}Cr (^{51}Sodium Chromate) was from Amersham (Toronto, Ontario). The following HLA-A2.1-binding and murine H-2K^b-binding peptides were used in the experiments: ILKEPVHGV (HIV rev), SLYNTVATL (HIV gag), YLSGANLNL (CEA tumor antigen CAP1 epitope), IMDQVPVSV, YLEPGPVTV, KTWGQYWQV (gp100 melanoma epitopes), NLVPMVATV (CMV pp65 epitope), DAPIYTNV (β-galactosidase H-2K^b epitope), TPHPARIGL ((β-galactosidase H-2L^d), and TLDSQVMSL (TRP-2 melanoma epitope). Peptides were synthesized in a 396 Multiple Biomolecular Synthesizer (Advanced Chemtech, Louisville, KY) and purified by HPLC. ALVAC-gal and ALVAC-gp100 were propagated in chick embryonic fibroblasts (CEF) and purified on a sucrose cushion by

centrifugation from lysed cells, as previously described (Ferrari et al., 1997). Viral titers in plaque-forming units (pfu) were determined in CEF, as previously described (Santra et al., 2002).

B. Mice

Balb/c and C57BL/6 mice were purchased from Charles River (Montreal, QC). HLA-A2.1/K^b transgenic mice were generated as described (Borenstein et al., 2000). All mice were house under specific-pathogen fee (SPF) conditions in micro-isolator cages in the Animal Resources Department of Aventis Pasteur (Sunnybrook Campus, Toronto, ON) adhering to Canadian Council on Animal Care (CACC) guidelines.

C. Human PBMC preparations

Human PBMC were obtained from leukopherisis donors using Ficoll-Hypaque gradient centrifugation (Sigma, St. Louis, MO). All blood products were collected at the Sunnybrook and Women's Health Sciences Center (Toronto, ON) according to institutional (IRB guidelines with complete donor consent. Donors were pre-screened for HLA-A*0201 using DNA sequencing and only positive individuals subjected to leukopherisis collections.

D. Murine MLR and preparation of effector and target cells

Spleens from C57BL/6 or Balb/c mice were harvested in mouse cell culture medium (α-MEM, 10% FCS, 50 μM mercaptoethanol, 1 mM Glutamax, Penicillin-Streptomycin) and cell suspensions were made using a stomacher machine (Stomacher 80 Biomaster, UK). The cell suspension was filtered through a 70 μm pore size strainer (BD Biosciences). The cells were washed and re-suspended in a culture medium. Stimulator cells were irradiated at 2000 Rads (Gammacell®1000 Elite, MDS Nordion) and washed once in culture medium and re-suspended in culture medium at 10 x 10^6 cells/ml. Responder cells (20 x 10^6 cells) and irradiated stimulator cells (20 x 10^6 cells) were placed into upright T-25 flasks in a total of 20 ml. The cultures were incubated for 5 days and viable cells harvested using centrifugation over Lympholyte-M (Cedarlane Labs, Canada) at 100xg for 20 min. The cells were washed in culture medium at 1.5 x10^6 /ml.

E. Mouse immunization studies and *in vitro* T-cell re-stimulation

Peptide immunization was performed using a melanoma H-2^b-binding TRP-2 tumour antigen peptide linked to a transcytosis peptide from the human Period 1 gene. This sequence induces the uptake of the TRP-2 sequence in APC and induces a strong anti-peptide CTL response. C57BL/6 mice were immunized with 50 μg of the TRP-2 peptide subcutaneously and boosted after 3 weeks. Three weeks after boosting spleens were harvested and spleen cells re-stimulated with TRP-2 peptide for 5 days and then subjected to CTL assays using either ^{51}Cr-release or using the caspase 3-cleavage assay. Immunization with a canarypox viral vector (ALVAC-gp100) was performed subcutaneously in HLA-A2.1/K^b transgenic mice (Borenstein et al., 2000). The mice were immunized with 2 x 10^7 pfu of ALVAC-gp100 followed by a booster at the same dose 2 weeks later. Splenocytes were harvested 5 weeks and 7 weeks after boosting and re-stimulated (5 x 10^6 cells/well in 24-well plates) with a pool of dominant HLA-A2.1-binding gp100 peptides (IMDQVPVSV, YLEPGPVTV, and KTWGQYWQV), at 0.2 μg/ml/peptide, for 5 days. The cells were harvested and subjected to analysis using IFN-γ ELISPOT or the caspase 3-cleavage assay. The ELISPOT analysis was performed with 100,000 re-activated spleen cells mixed with 10,000 gp100 peptide-pulsed (2 μg/ml) P815 stably transfected with an HLA-A2/K^b expression plasmid (P815-A2/K^b cell line).

F. Generation of human peptide-specific T cells

T-cell-enriched PBMC (plastic-adherent negative fraction of PBMC) were stimulated using peptide-pulsed autologous mature dendritic cell (DC) in human T-cell culture medium (HTC-CM) consisting of Iscove's Modified Dulbecco's Medium, 1 mM Glutamax, 1 mM pyruvate (Invitrogen, Mississauga, ON), 5% human AB serum, 50 μM 2-mercaptoethanol, 20 μg/ml gentamycin. Mature DC were generated in a one-step protocol over 6 days from adherent monocytes using HTC-CM containing 2% human AB serum, 1,000 U/ml GM-CSF, 1,000 U/ml IL-4, 4 ng/ml TNF-α, and 100 U IFN-α. The DC were harvested, washed and pulsed with 10 μg/ml of peptide. The following HLA-A2.1-binding nonamer peptides were used for T-cell generation: HIV rev peptide (ILKEPVHGV), HIV gag peptide (SLYNTVATL) and the CAP1 epitope from the CEA tumor antigen (YLSGANLNL). These peptides have been previously demonstrated to bind to HLA-A2.1 and elicit HLA-A2.1-

restricted $CD8^+$ T-cell responses (Stuber et al., 1992; Herr et al., 1996; Zaremba et al., 1997). The pulsed DC were washed and used to stimulate T-cell-enriched autologous PBMC (T cells) in 24-well plates at a ratio of 0.6 x 10^6 pulsed DC with 6 x 10^6 T cells. IL-2 (10 U/ml), IL-7 (5 ng/ml), IL-6 (1,000 U/ml), and IL-12 (10 ng/ml) were added at culture initiation and the cells fed with IL-2 (additional 10 U/ml) after 4 days. Primary activations were performed for 10 to 12 days followed by 2 to 3 rounds of re-stimulation using peptide-pulsed CD40 ligand-activated autologous B lymphocytes, generated as previously described (Schultze et al., 1997). Effector cell assays (caspase 3-cleavage, IFN-γ ELISPOT, CTL assays using Annexin-V/7-AAD, and HLA-pentamer staining) were performed 5 days after the third or fourth stimulation. In some cases, the activated T cells were diluted with naïve autologous PBMC before the assays, as indicated.

G. Caspase 3-cleavage assay and staining methodology

*1. **Target cell labeling and peptide pulsing.*** Harvested target cells (P815, EL4, T2 lymphoma cells) were washed once with D-PBS. The cells were re-suspended at 5 x 10^6/ml in labeling buffer containing 0.6 μM DDAO-SE (Molecular Probes, Eugene, OR) in D-PBS and incubated at 37°C for 15 min. The cells were washed in culture medium and re-suspended in culture medium at 2 x 10^6 cell/ml and pulsed with the indicated peptides at 1-3 μg/ml for 1 h. The pulsed targets were washed once in culture medium and re-suspended at 1 x 10^6 cells/ml.

*2. **CTL assay set-up and antibody staining.*** DDAO-SE-labeled target cells pulsed with peptide (0.1 ml) were mixed with 0.1 ml of effectors cells (1 x 10^6 cells/ml) in conical polypropylene Costar cluster tubes (Costar, Corning, NY). The cell mixture was centrifuged at low speed (200 rpm) for 1 minute. The mixtures were incubated at 37°C, 5% CO_2 in a humidified incubator for the indicated time periods (0.5 to 5 h). The cells were washed with D-PBS, 1% BSA at room temperature (RT) and either fixed and permeabilized with Fix/Perm solution (BD Biosciences, Mississauga, ON) 20 min at RT immediately or fixed in 1% paraformaldehyde for 20 at RT and then stored at 4°C for up to 24 h. Fixed and stored cells were centrifuged and re-suspended in Fix/Perm buffer at RT for 20 min. Cells were then washed 2 times with staining buffer (D-PBS, 1% BSA, 0.1% saponin) and re-suspended in 0.1 ml staining buffer. The cells were stained for 60 min on ice with 15 μl biotin-labeled

anti-cleaved caspase 3 monoclonal antibody (BD Biosciences, Mississauga, ON). The cells were washed in staining buffer and counter-stained with Strepavidin-PE (Sigma, St. Louis, MO) for 30 min. on ice. The cells were washed in staining buffer 2 times and re-suspended in D-PBS, 1% BSA for analysis on a flow cytometer.

3. ***Flow cytometric analysis.*** The stained cells were analyzed in a FACSCalibur flow cytometer (BD Biosciences, Mississauga, ON). Thirty to 50 thousand events were collected for each sample. Live cells and target cells were gated using the forward and side scatter parameters followed by gating of the DDAO-SE-labeled target cell population on the FL4 channel (see also Fig. 1B). Cleaved caspase 3 expression (FL2 channel) was then determined using the DDAO-SE gated target cells in an FL4 versus FL2 dot plot.

H. IFN-γ ELISPOT assay

ELISPOT essay was using Millipore MultiScreen-HA 96-well filter plates (Millipore Cat No. MAHAS4510). Plates were coated with 5 μg/ml anti-IFN-γ mAb in pH 9.5 carbonate buffer (Sigma, St. Louis, MO) overnight at 4°C and blocked with D-PBS, 2% BSA. Human or murine T cells (100,000/well) were added, as indicated and incubated with relevant or irrelevant peptides overnight. The plates were washed and incubated with biotin-labelled anti-IFN-γ mAb for 1 h at RT. The plates were washed and then treated with 1:5,000 dilution of Extravidin-Alkaline phosphatase (Sigma) for 1 h at RT. The plates were developed with BCIP/NBT substrate (Sigma) until all spots were clearly visible. Developed plates were dried and counted on an AID Version 3.1.1 ELISPOT reader (Cell Technologies, USA). Cells treated with PMA and Ionomycin (Sigma) were used as positive controls in all experiments.

I. ^{51}Cr-release Assay

Target cells (P815, P815-A2/K^b, EL4, and T2 cells) were labeled with 100 μCi $Na_2{}^{51}CrO_4$ in cell culture medium containing 10% FCS for 45 min at 37°C. The cells were washed in culture medium and pulsed with the specific or non-specific control peptides (1 to 3 μg/ml) for 1 h if the protocol required. The pulsed cells were washed once in culture medium and re-suspended at 30,000 cells/ml. Effector cells (murine MLR, peptide re-stimulated spleen cells, or peptide-activated human T-cell lines) were added at different E:T ratios, as indicated in the results. In some experiments,

the effector cells were diluted with naïve lymphocytes before addition at various E:T ratios to the ^{51}Cr-release assay. After 4 h incubation, 25 µl of the assay supernatant was placed into a 96-well Lumaplate (Packard). The plates were dried and radioactivity counted using a TopCount NXT v2.12 scintillation counter (Packard). The results are expressed as % specific lysis calculated as (Experimental release – Spontaneous release / Total release – Spontaneous release) x 100.

J. HLA-pentamer staining

HIV gag peptide-specific and CEA CAP1 peptide-specific HLA-A*0201 human T-cell lines were stained with PE-conjugated recombinant HLA-peptide pentamers (ProImmune, Oxford, UK). An HLA-A*0201 pentamer containing the SLYNTVATL sequence from HIV gag (ProImmune code 010) was used to stain the HIV-specific lines, while an HLA-A*0201 pentamer containing the YLSGANLNL peptide from CEA (ProImmune code 075) was used to stain the CAP1 peptide-specific line. In both cases, an HLA-A*0201-restricted T-cell line consisting of a similar percentage of $CD8^+$ T-cells was used as a non-specific pentamer staining control. The T cells were washed and re-suspended at 2 x 10^6 cell/ml in pentamer staining buffer (PSB) consisting of D-PBS, 0.1% NaN_3, 0.1% BSA. The cells were stained with 10 µl Pro5™ for 20 min at RT, washed and then stained in PSB with 5 µl anti-human CD8-FITC (BD Biosciences) for 20 min at RT. The cells were washed in PSB and fixed in D-PBS, 1% paraformaldehyde and run through a FACScalibur flow cytometry analyser. The pentamer-positive cells were detected and quantitated by gating first on live cells using the forward scatter and side scatter discriminators and then analysing on a two-colour plot showing $CD8^+$ fluorescence on the x-axis and $pentamer^+$ fluorescence on the y-axis. Results are shown as % $pentamer^+$ cells for each culture.

Example 2

A. Assay procedure and choice of target cell tracking dye

One of the first issues we resolved is the problem of finding an effective target cell tracking dye that would not interfere with the caspase 3 signal during FACS analysis. To maximize sensitivity, we wanted to use a biotin-labeled primary anti-cleaved caspase 3 followed with a Strepavidin-PE counter-stain brightly fluorescing in the FL2 channel. As a result, we searched for a non-toxic cell tracker dye that

would fluorescence in the far-red (FL4) channel and not require any compensation with the FL2 channel. We found that DDAO-SE (Molecular Probes) met these requirements. As shown in Fig. 1A, DDAO-SE was found to reproducibly and stably stain of a variety of murine and human cell lines for up to 15 h, including P815, EL-4, B16F10 melanoma, 4T1 breast cancer, human T2 thymoma, and COS cells, with no induction of non-specific caspase 3 cleavage.

Using DDAO-SE-stained target cells, we first tested the caspase 3-cleavage assay to measure CTL activity in murine allogeneic T-cell responses. Mixed lymphocyte responses of C57BL/6 anti-Balb/c and Balb/c anti-C57BL/6 spleen cells were tested after 5 days of culture in comparison to ^{51}Cr-release. Effector cells were mixed with target cells (P815 or EL-4 cells) at different ratios and incubated for different time intervals and fixing and stained with anti-cleaved caspase 3. Fig. 1B shows the gating strategy employed top enumerate the percentage of caspase 3-cleaved target cells. This approach was used in all experiments. Forward scatter (FSC) and side scatter (SSC) plots were used to isolate the population of live DDAO-SE$^+$ (FL4 channel) target cells as shown (R2 and R3). The R2 and R3 gates were combined and used to determine the percentage DDAO-SE$^+$, caspase 3-cleaved (FL2 channel) targets. This gating strategy ensured that only live targets were taken into consideration.

Experiments with C57BL/6 anti-Balb/c MLR effector cells with P815 targets (1:1 E:T ratio) found that induction caspase 3- cleavage was specific for H-2^d P815 targets and no marked cleavage was detected in H-2^b EL4 targets (Fig. 2A). The time course shown in Fig. 2A and 2B also shows that the caspase 3 cleavage signal was maximal after 1 h incubation in this case. Similar results were found when Balb/c anti-C57BL/6 MLR effector cells were tested (Fig. 2B). Overall, the caspase 3-cleavage assay exhibited the same specificity as using a 4-hour ^{51}Cr-release assay to measure allo-specific CTL activity in the MLR (data not shown).

In order to confirm that the cleavage of caspase 3 in target cells was dependent on cytotoxic granules secreted by CTL, we incubated C57BL/6 anti-Balb/c MLR effector cells with Concanamycin A, used as a CTL degranulation inhibitor in CTL assays, before mixing with the targets. Concanamycin A inhibited the appearance of caspase 3 cleavage in the P815 target cells (Fig. 2C). Similar results were obtained with an assay performed using human CTL line reactive against the CAP1 peptide from the CEA tumor antigen (data not shown). In addition, inhibition of caspase 3

activity using the caspase inhibitor, Z-DEVD-FMK, or a general caspase inhibitor, Z-VAD-FMK, did not prevent the appearance of cleaved caspase 3 in the P815 targets of the C57BL/6 anti-Balb/c MLR (data not shown). Together, these results indicate that caspase 3 cleavage in target cells is dependent on granzymes secreted by the CTL and is not triggered by endogenously activated caspase 3, or other caspases cleaving caspase 3 in the target during the killing process.

The caspase 3-cleavage assay was compared to the ^{51}Cr-release assay in two different assay systems, the murine C57Bl/6 anti-Balb/c MLR system and in a peptide vaccination response in HLA-A2.1/K^b transgenic mice (Fig. 3). In the MLR model, the caspase 3-cleavage assay showed the same specificity and reliability as the ^{51}Cr-release assay. However, it was markedly more sensitive being able to detect CTL activity at E:T ratios below 0.5:1 (Fig. 3A). Spleen cells from mice vaccinated with a human TRP2 melanoma antigen peptide were also tested for CTL activity against peptide-pulsed P815-A2/K^b target cells (Fig. 3B). The caspase 3-cleavage assay yielded similar results as ^{51}Cr-release when peptide re-stimulated spleen cells were tested at a 40:1 and 10:1 E:T. Again, as before, the assay showed a significantly higher degree of sensitivity with higher rates of specific CTL activity detected at the 10:1 E:T ratio (Fig. 3B).

B. Use of caspase 3-cleavage assay to monitor CTL responses against viral vector vaccines

The caspase 3 cleavage CTL assay was compared to the IFN-γ ELISPOT assay to measure gp100 peptide-specific the CD8$^+$ recall response following immunization of HLA-A2.1/K^b transgenic mice with 2 x 10^7 pfu ALVAC-gp100. The mice were primed and boosted and spleen cells re-stimulated with 0.2 μg/ml of a pool of HLA-A2.1-binding gp100 peptides for 5 days and then subjected to effector cell assays using either caspase 3-cleavage or IFN-γ ELISPOT. The caspase 3-cleavage assay used DDAO-SE-labeled gp100 or control peptide-pulsed P815 cells stably transfected with HLA-A2.1/K^b plasmid. A similar approach was used in the ELISPOT assays, except the P815-A2/K^b cells were not DDAO-SE labeled (see methods section). Five replicate mice were used in each assay to test the precision of the results between individual animals when using the caspase 3-cleavage assay as a read-out. Fig. 4 shows the results of two separate experiments comparing the caspase

3-cleavage read-out for gp100 peptide-specific $CD8^+$ T-cell responses versus ELISPOT. Both assays yielded comparable results with no non-specific reactivity in mice immunized with an empty ALVAC control vector. The scatter of between individual animals was low indicating that monitoring $CD8^+$ T-cell responses using caspase 3-cleavage in CTL targets can accurately measure antigen-specific $CD8^+$ T-cell responses post-vaccination with high precision.

These results indicate that measurement of caspase 3 cleavage in CTL target cells in murine systems is a viable alternative assay to ^{51}Cr release and has the same degree of specificity but is markedly more sensitive that ^{51}Cr release. In addition, measurement of CTL activity via caspase 3 cleavage can also be used as a supportive functional assay for IFN-γ ELISPOT or as a substitute assay for the ELISPOT when target cells expressing the target antigen or peptide are available.

C. Caspase 3-cleavage CTL assay for monitoring antigen-specific human T cell responses

In a first set of studies, human T cell lines were generated from HLA-A*0201$^+$ donors against a 9-mer HIV rev peptide, an HIV gag peptide, or against the CAP1 peptide from the tumor-associated antigen CEA. All T-cell lines were generated from normal, non-HIV-infected and non-cancerous donors by repeated cycles of peptide stimulation and proliferation with IL-2 and IL-7 as outlined in the methods. After the third or fourth stimulation cycle, we routinely acquire T-cell lines consisting of >85% $TCR\alpha\beta^+$, $CD8^+$, $CD16^-$, $CD56^-$ proliferating T cells. These T cells were tested for effector cell activity using monitoring caspase 3-cleavage. IFN-γ ELISPOT analysis confirmed the presence of peptide-specific T cells after the third and fourth round of stimulation. These ranged from 1:1 00 to 1:250 for both the HIV peptide-specific and CEA peptide-specific lines (data not shown).

Fig. 5A shows typical results of the caspase 3-cleavage assay for monitoring the activity of activated human T cells specific using an HLA-A2.1-restricted T-cell line specific for a peptide from HIV rev. The activated T cells were mixed with pulsed T2 targets at a 5:1 or 1:1 E:T ratio (Fig. 5A). The assay proved to be specific with only minimal level of caspase 3 cleavage in T2 targets pulsed with a non-specific HLA-A*0201-binding peptide from the pp65 CMV antigen. In Fig. 5B, we compared the results of the caspase 3-cleavage assay to that of another FACS-based CTL assay

that detects apoptosis in targets using Annexin-V and 7-AAD staining (Fischer et al., 2002) with a kit (BD Bisciences) using T-cell lines specific for the CAP1 HLA-A2.1 epitope of CEA. The T-cell lines (4^{th} stimulation) were diluted with naïve autologous PBMC from the donor at the ratios indicated and mixed with an equal number (100,000) peptide-pulsed T2 target cells. Both assays yielded comparable results with a similar level sensitivity (Fig. 5B). However, we noticed in a number of experiments that the Annexin-V + 7-AAD assay had a significantly higher non-specific background. In addition, we found that there was considerable variation in level of Annexin V fluorescence between samples (data not shown). Annexin V binding to phosphatidyl serine is Ca^{2+} sensitive and slight alterations in Ca^{2+} concentration can alter the Annexin-V binding equilibrium. This variation in staining fluorescence was not found when staining for caspase 3-cleavage.

In addition to the lower background, the caspase 3-cleavage assay exhibited a significant degree of sensitivity in tracking CTL activity, as shown by the detection of specific CTL activity at the 1:7 dilution of effector cells (Fig. 5B; Caspase 3 cleavage). We also determined the frequency of CAP1-specific T cells in the T line used in Fig. 5B with the IFN-γ ELISPOT assay (not shown) and found a 1:237 frequency. Based on these results, we estimated that the frequency of CAP1-specific T cells at the 1:3 and 1:7 dilutions in Fig. 5B was 1:711 (0.14%) and 1:1659 (0.06%), respectively. This suggests that the assay is sensitive and capable of detecting rare antigen-specific $CD8^+$ T cells, especially against TAA where CTL frequencies can be quite low.

D. Sensitivity of the caspase 3-cleavage-based CTL assay

To further tests the sensitivity limits of the assay, a series of experiments were performed with mouse MLR effector cells and activated peptide-specific human T cell lines in which the effector cells were diluted up to 1:199 with naïve syngeneic spleen cells or with naïve autologous PBMC, respectively.

The first experiment was performed with C57BL/6 anti-Balb/c MLR effectors cells diluted after harvesting the MLR at 1:0 (no dilution) to 1:199 with naïve syngeneic C57BL/6 splenocytes (Fig. 6). The diluted effectors were incubated with DDAO-SE-labelled P815 cells at a 1:1 ratio. Target cells alone, and naïve splenocytes served as negative controls for CTL activity and DDAO-SE-labeled EL-4

targets served as specificity controls. The caspase 3-cleavage exhibited a high degree of sensitivity, as shown by the presence of significant caspase 3 cleavage even when the MLR effector cells were diluted at a ratio of 1:199 with naïve splenocytes (Fig. 6). An insignificant level of caspase 3 cleavage was seen at all dilutions in the non-specific EL4 targets (Fig. 6). The percentage of allo-reactive $CD8^+$ splenocytes in a typical murine MLR has been found to be as high as 1:10. Thus, assuming that this frequency of anti-Balb/c effectors was present in the MLR, at the 1:199 dilution with naïve splenocytes the assay was able to detect CTL activity at a 1:2,000 (0.05%) frequency of effector cells. This experiment was repeated at least 3 times with similar results.

A similar dilution experiment was performed with HIV peptide-specific human HLA-A*0201-restricted T cell lines after 3 to 4 rounds of stimulation from a naïve PBMC pool (Fig. 7). In the experiment shown in Fig. 9A, IFN-γ ELISPOT analysis estimated that the frequency of HIV peptide-specific T cells in the cultures was 1:156 (0.64%) after 3 stimulations. These T cell cultures were diluted with naïve autologous PBMC at a 1:0 to 1:199 ratio. As before, DDAO-SE-labeled T2 lymphoma cell targets alone and naïve PBMC minus activated T cells incubated with the HIV peptide-pulsed T2 targets were used negative controls, while T2 cells pulsed with the HLA-A*0201-binding pp65 CMV peptide was used as a control for specificity. As shown in Fig. 7B and 9B, measurement of caspase 3-cleavage was remarkably sensitive detecting significant activity at effector cell dilutions as low as 1:99 and 1:199. A rate of caspase 3 cleavage of 5.7% and 6% was seen at 1:99 and 1:199, respectively, in the HIV peptide-pulsed T2 cells, while only 2.1% and 2.2% caspase 3 cleavage was seen in the non-specific pp65 CMV peptide-pulsed targets. This low percentage of caspase 3-cleaved cells with the CMV peptide did not increase as the dilution of effector cells decreased. The percentage of caspase 3 cleavage in the targets minus the effectors and with naïve PBMC plus targets was less than 2% (Fig. 7B). As a further test of the sensitivity of the assay, we also stained the HIV gag-specific T cell lines with an HLA-peptide pentamer (ProImmune, Oxford, UK) that directly stains the TCR of the antigen-specific T cells (Fig. 7C). Fig. 7C shows the results of the pentamer staining on the same T cells used in the caspase 3-cleavage assay shown in Fig. 7B. This analysis revealed that the caspase 3-cleavage assay had a comparable level of sensitivity as HLA pentamer staining. The frequency of HIV

gag peptide-specific T cells in the caspase 3-cleavage assay (Fig. 7B), based on the IFN-γ ELISPOT frequency, was 1:15, 444 (0.007%) and 1:31,044 (0.003%) for the 1:99 and 1:199 dilutions of the effectors, respectively. This represents a high level of sensitivity making the assay useful for quantitating CTL activity when antigen-specific T-cell frequencies are considerably low. Moreover, unlike pentamer- or tetramer- based methods, it is a measure of T cell function and not only T-cell frequency.

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